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Vaccination with Genetically Modified Shiga-Like Toxin IIe Prevents Edema Disease in Swine

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***Escherichia coli* strains producing Shiga-like toxin II variant (SLT-IIe, formerly called SLT-IIv) cause edema disease in weaned pigs. Vaccination of pigs with a genetically modified form of Shiga-like toxin IIe, SLT-IIe(E167Q), has been previously shown to be nontoxic and to induce antibodies to SLT-IIe (V. M. Gordon, S. C. Whipp, H. W. Moon, A. D. O'Brien, and J. E. Samuel, Infect. Immun. 60:485–502, 1992). Fifty micrograms of SLT-IIe(E167Q) toxin was used to vaccinate suckling pigs at 1 and 2 weeks of age. Both vaccinated and nonvaccinated pigs were orally inoculated with an SLT-IIe-producing strain of *E. coli* after weaning (3 to 4 weeks of age). Pigs fed a low-protein diet that were not vaccinated with SLT-IIe(E167Q) developed subclinical edema disease, histologically evident as vascular necrosis. Pigs fed a high-protein diet that were not vaccinated with SLT-IIe(E167Q) developed clinical edema disease manifested as vascular necrosis, reduced weight gain, ataxia, palpebral edema, lateral recumbency, and death. Pigs vaccinated with SLT-IIe(E167Q) had a reduction in the incidence of subclinical edema disease and never developed clinical edema disease. These data demonstrate that vaccination with a genetically modified form of SLT-IIe prevents edema disease and are consistent with the notion that diet influences susceptibility to edema disease.**

Escherichia coli strains producing one or more cytotoxins in the family of Shiga-like toxins (SLTs) have been implicated as the cause of hemorrhagic colitis and hemolytic-uremic syndrome in humans (17–20, 29, 33), diarrhea in calves (7, 25, 34), and edema disease in swine (8, 10, 26, 35). These toxins are bipartite toxins consisting of five B subunits responsible for binding to specific cell receptors and an A subunit containing the enzymatic activity (28). The activity of the A subunit inhibits protein synthesis, resulting in cytotoxicity and the clinical syndromes cited above (9, 14, 30). The specificity of the clinical syndromes attributable to the different SLTs is thought to be a function of differing receptor specificities (31).

The clinical manifestations of edema disease include palpebral edema and neurologic dysfunction manifested as ataxia, lateral recumbency, and death (27, 37). These signs result from microangiopathy and vascular necrosis caused by a variant of Shiga-like toxin II (SLT-IIe, formerly called SLT-IIv) (10, 23, 26). Subclinical edema disease is characterized by microangiopathy in animals that do not manifest the signs cited above and has been observed to occur in experimentally infected animals (22). Diet and other factors influence the susceptibility of pigs to *E. coli* producing SLT-IIe (27) and could determine whether subclinical or clinical edema disease occurs following infection.

There are several lines of evidence that antitoxic immunity protects against diseases caused by *E. coli* producing Shiga-like toxins. In 1955, Howard observed that shigella dysentery antitoxin could protect mice and rabbits against lethal injections of Shiga toxin (13). More recently, similar protection was achieved in mice which had received a monoclonal antibody specific for Shiga toxin (16). Passive protection of mice experimentally infected with SLT-II-producing *E. coli* was also demonstrated with anti-SLT-II monoclonal antibody (38). Awad-Masalmeh et al. (1, 2) vaccinated pigs with a toxoid prepared by exposure of crude SLT-IIe to glutaraldehyde and reported protection against naturally occurring and experimentally induced edema disease. MacLeod and Gyles (24) subsequently inactivated purified SLT-IIe by exposure to glutaraldehyde and demonstrated that passive transfer of antibodies against, or intramuscular vaccination of 2-week-old pigs with, this product protected pigs against a lethal intravenous dose of SLT-IIe.

Previously, a toxoid prepared by chemically modifying SLT-IIe with 1% formaldehyde was tested for efficacy against subclinical edema disease (11). Even though this preparation had no detectable cytotoxic activity in Vero cells, pigs vaccinated with this SLT-IIe toxoid had a reduced rate of gain in comparison with littermate controls (11). Since the presence of glutamate at position 167 of the mature A subunit of SLTs was implicated as being important for enzymatic activity with both SLT-I and SLT-II (12), SLT-IIe toxin was modified by site-directed mutagenesis and tested as a potential vaccine to prevent edema disease (11). This genetically modified SLT-IIe toxin differed from the parent toxin with respect to one amino acid: the glutamate at position 167 was changed to glutamine (E167Q). The cytotoxic activity and lethality to mice of SLT-IIe(E167Q) were reduced 10^6- and 10^4-fold, respectively, in comparison with those of the native toxin. Moreover, vaccination of pigs with SLT-IIe(E167Q) did not have deleterious effects on rate of gain (11).

The primary objective of the present study was to determine if vaccination of pigs with SLT-IIe(E167Q) could prevent both subclinical and clinical edema disease. Initially, SLT-IIe(E16

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7Q) was tested by using a previously described subclinical edema disease model (22). This model was modified, and clinical edema disease resulted when the protein content of the diet was increased. SLT-IIe(E167Q) was also tested in this experimental model of clinical edema disease. These experiments demonstrated that vaccination with SLT-IIe(E167Q) markedly reduced the incidence of both subclinical and clinical edema disease.

MATERIALS AND METHODS

Bacterial strains. *E. coli* S1191 (serogroup O139), originally isolated from a pig with clinical edema disease, and 123 (serogroup O43), originally isolated from a clinically normal pig, were used in principals and controls, respectively. Strain S1191 is hemolytic and produces SLT-IIe, heat-stable enterotoxin b, and the F107 fimbriae which mediate colonization of the swine intestine (3, 15). Strain 123 is nonhemolytic, nontoxigenic, and nonpathogenic.

Mutant toxin vaccine. The mutant toxin SLT-IIe(E167Q) was produced by site-directed mutagenesis to replace glutamate at position 167 with glutamine to reduce the enzymatic activity of the A subunit, as previously described (11).

Animal experiments. Three different experimental models were used to determine if diet and inoculation method affected severity of edema disease. In all three models, swine were vaccinated with the modified SLT-IIe(E167Q) protein to determine the ability to induce protective immunity against an edema disease-causing strain of *E. coli.*

Model A. Model A has been previously used to induce subclinical edema disease (11). One hundred and two naturally farrowed piglets from 12 crossbred litters were randomly assigned to four groups. Two sham-vaccinated groups were injected subcutaneously with 1 ml of $A1(OH)_3$ adjuvant-phosphate-buffered saline (1:1), and the two vaccinated groups were injected subcutaneously with 1 ml of adjuvant containing 50 μg of SLT-IIe(E167Q). Pigs were vaccinated at 7 and 14 days of age and weaned at 3 weeks of age, and fasted on the night of weaning. Following the overnight fast, pigs were inoculated once intragastrically with 10^{10} CFU of *E. coli* and then fed a commercial ration containing 18.0% crude protein ad libitum. Nonvaccinated ($n = 26$) and vaccinated ($n = 26$) principals were inoculated with edema disease-causing *E. coli* S1191. Nonvaccinated ($n = 25$) and vaccinated ($n = 25$) controls were inoculated with the nonpathogenic *E. coli* 123.

Model B. Because model A demonstrated that vaccinated and unvaccinated controls challenged with strain 123 behaved similarly, only one group of controls was used in models B and C. Thirty-four pigs from five crossbred litters were randomly assigned to three groups. Pigs were weaned at 3 weeks of age and fed a commercial ration which contained 17.0% crude protein ad libitum. Vaccinated principals ($n = 11$) were inoculated per os at 4, 6, 8, and 10 days postweaning with gelatin capsules containing approximately 1 g of feed and 10^{10} CFU of strain S1191. The control group ($n = 11$) was similarly inoculated with strain 123. Nonvaccinated principals ($n = 12$) were similarly inoculated with strain S1191. Feed consumption was monitored daily.

Model C. Thirty-six pigs from six litters were randomly assigned to three groups and vaccinated and inoculated as in model B. All pigs in the model were fed a commercial ration containing 21.0% crude protein ad libitum. The vaccinated ($n = 8$) and unvaccinated ($n = 17$) principals were inoculated with edema disease strain S1191 four times, as in model B. Eleven control pigs were similarly challenged four times with the nonpathogenic strain 123. Feed consumption was monitored daily.

Clinical observations, postmortem, and serology. All pigs were weighed daily and observed for clinical signs of edema disease (palpebral edema, ataxia, lateral recumbency, or death). Any animal in lateral recumbency or exhibiting ataxia for 48 h was euthanized. Pigs were necropsied between 12 and 17 days after the initial challenge. Spiral colon, ileum, and brain stem specimens were collected and immersed in neutrally buffered 10% formalin. Histologic sections were prepared as described previously (22), stained with hematoxylin and eosin, coded, and then examined by light microscopy. The histologic sections were examined in a double-blind fashion. Animals were designated positive for vascular necrosis if one or more of the tissues examined had at least two vessels with vascular necrosis. All animals were bled at the time of the first challenge and at necropsy. Serum antibodies against SLT-IIe were determined by serum neutralization of SLT-IIe Vero cell cytotoxicity as described previously (11).

Statistical analysis. The rates of gain of the various treatment groups after challenge were compared by using one-way analysis of variance (32) and were calculated from linear regression for individual pigs in each treatment group. Weight gain of pigs euthanized or found dead early in the experiment was not included. The ratio of daily feed consumption/weight gain was determined only for models B and C and was calculated by dividing daily feed consumption (kilograms) of a treatment group by daily weight gain (kilograms) of all pigs in that treatment group. The ratios of average daily feed consumption/weight gain for treatment groups were compared by one-way analysis of variance (32).

TABLE 1. Effect of diet and number of inoculations on prevalence of clinical edema disease

Model	Diet (% protein)	No. of inoculations	No. with clinical edema disease[a]
A	18	1	0/26
B	17	4	0/12
C	21	4	10/17

[a] Number of nonvaccinated principals with clinical edema disease/total number of nonvaccinated principals. No clinical edema disease was observed in any of the controls or vaccinated principals.

RESULTS

Model A. All model A pigs were inoculated once with either the nonpathogenic strain 123 (controls) or the edema disease-causing strain S1191 (principals). No clinical signs of edema disease were observed in any pig (Table 1). However, microangiopathy consistent with edema disease was observed in 9 of 26 nonvaccinated principals. Such histologic lesions were observed in only 2 of 26 vaccinated principals. No such lesions were observed in 25 nonvaccinated or 25 vaccinated controls.

Nonvaccinated principals had a slight, but not significant ($P = 0.09$), decreased rate of gain relative to those of the other three treatment groups (Fig. 1). Thirty-five of 49 pigs vaccinated with SLT-IIe(E167Q) and 0 of 51 nonvaccinated pigs developed toxin-neutralizing serum antibody titers greater than 1:4 prior to challenge. Titers of antibody to SLT-IIe in serum increased after challenge in vaccinated pigs inoculated with strain 123 as well as in those inoculated with strain S1191 (Table 2).

Model B. Model B animals were challenged four times with either nonpathogenic strain 123 (controls) or edema disease strain S1191 (principals) to determine if multiple inoculations could induce clinical edema disease. No clinical signs of edema disease were observed in any pig (Table 1). Watery diarrhea was noted 6 to 11 days postweaning in all vaccinated and nonvaccinated principals but not in controls. Microangiopathy consistent with edema disease was noted in 6 of 12 nonvaccinated principals. No such lesions were observed in the 11 controls or the 11 vaccinated principals.

The nonvaccinated principals had a slight, but not significant ($P = 0.18$), decreased rate of gain relative to those of the other two groups (Fig. 2). The ratios of average daily feed consumption/weight gain of the three treatment groups were not significantly different. Animals vaccinated with SLT-IIe(E167Q) developed a median serum antibody titer of 1:16, which likely conferred the protection from subclinical edema disease (Table 2).

Model C. Multiple inoculations with edema disease strain S1191 increased the incidence of subclinical edema disease in model B animals relative to model A animals; however, no animals developed clinical edema disease. Therefore, an additional modification was made to enhance the ability to generate clinical edema disease in model C. Animals were fed a high-protein diet (comparable to commonly used commercial weaning rations) and inoculated four times with either nonpathogenic strain 123 (controls) or edema disease strain S1191 (principals). Clinical signs of edema disease were observed to be present in 10 of the 17 nonvaccinated principals (Table 1). Four pigs in this group developed clinical signs of edema disease (rear-leg ataxia and/or palpebral edema) and recovered. Six pigs in this group developed clinical signs of edema disease and did not recover. One died of edema disease, three were in lateral recumbency and were euthanized, and two were ill (rear-leg ataxia) for 2 days and were euthanized. Sixteen of the

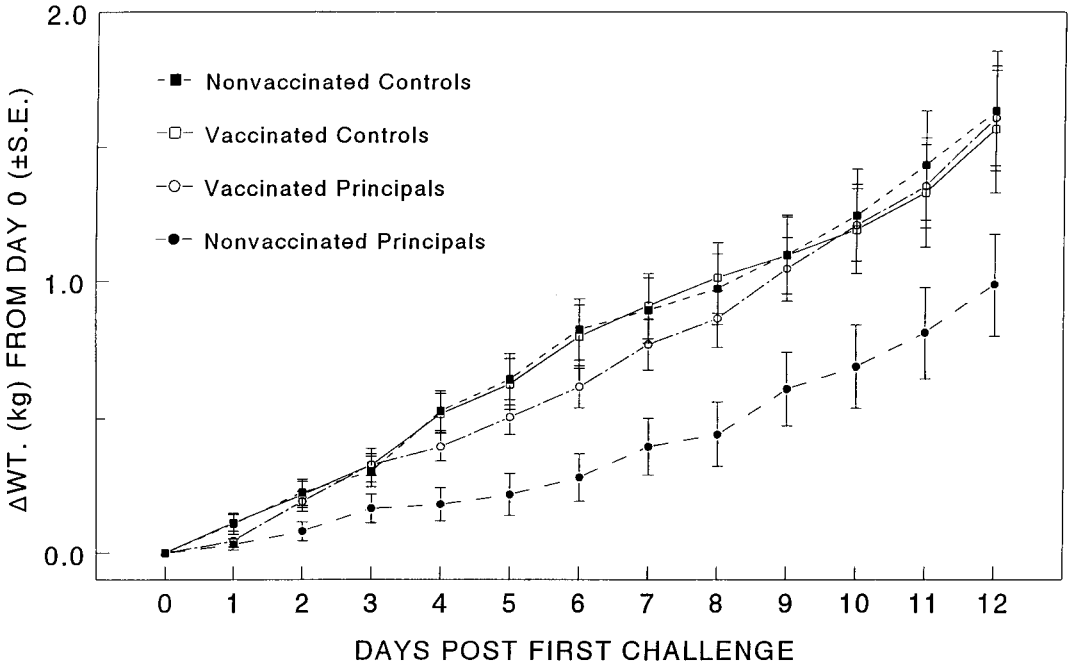

FIG. 1. Effect of vaccination with SLT-IIe(E167Q) and subclinical edema disease on rate of gain in pigs fed an 18% protein diet and inoculated one time. Pigs were either vaccinated with 50 μg of mutant toxin or not vaccinated at 1 and 2 weeks of age. Vaccinated (n = 26) and nonvaccinated (n = 26) principals were inoculated intragastrically 1 day postweaning with 10^{10} CFU of strain S1191. Vaccinated (n = 25) and nonvaccinated (n = 25) controls were similarly inoculated with strain 123. S.E., standard error.

17 pigs in this group had microangiopathy consistent with edema disease. No clinical signs of edema disease or vascular necrosis were observed in the 11 controls or in the 8 vaccinated principals.

Watery diarrhea was noted 5 to 9 days postweaning in all nonvaccinated and vaccinated principals but not in the controls. Vaccinated pigs had a median serum antibody titer of 1:16 following vaccination, and this likely conferred protection against clinical edema disease (Table 2). Antibody titers increased following inoculation with either strain 123 or strain S1191. Nonvaccinated principals had a significantly lower rate of gain relative to those of the other two treatment groups (Fig. 3) ($P < 0.001$). Nonvaccinated principals had a ratio of average daily feed consumption/weight gain (± standard deviation) of 2.44 (± 1.72), which was significantly different from results for the other two groups ($P < 0.05$). The ratios of average daily feed consumption/weight gain were 1.34 (± 0.53) for controls and 1.42 (± 0.56) for vaccinated principals.

TABLE 2. Anti-SLT-IIe titers in vaccinated and nonvaccinated pigs

Model	Group	Anti-SLT-IIe titer[a]		
		Prevaccination	Prechallenge	Postchallenge
A	Nonvaccinated controls	0	0	0
	Vaccinated controls	0	32	128
	Nonvaccinated principals	0	0	0
	Vaccinated principals	0	32	128
B	Nonvaccinated principals	0	0	0
	Vaccinated principals	0	16	32
	Vaccinated controls	0	16	16
C	Nonvaccinated principals	ND[b]	0	0
	Vaccinated principals	ND	16	64
	Vaccinated controls	ND	16	64

[a] Median titer for each group expressed as the minimum dilution that neutralized at least 50% of the cytotoxic activity of 10 Vero 50% cytotoxic dose units of SLT-IIe.

[b] ND, not determined.

DISCUSSION

Vaccination of suckling piglets with the mutant toxin SLT-IIe(E167Q) subsequent to challenge with edema disease strain S1191 markedly decreased the incidence of vascular lesions in all three models. This was consistent with the evidence indicating that such lesions are caused by SLT-IIe (4, 11). The results obtained with model A confirm a previous report that a single inoculation of weaned pigs with *E. coli* S1191 results in subclinical edema disease manifested as vascular necrosis. However, vascular necrosis occurred less frequently in model A in this study (9 of 26 nonvaccinated principals) than reported previously (20 of 21 pigs) (22). Increasing the number of inoculations with strain S1191 to 4 in model B versus 1 in model A slightly increased the incidence of vascular necrosis (6 of 11 versus 9 of 26 nonvaccinated principals), but no clinical edema disease was observed. Clinical edema disease was only seen in model C in 10 of 17 unvaccinated principals challenged four times with strain S1191 and fed a high-protein diet. All but 1 of the 17 pigs in this group developed vascular necrosis.

Model C was the best model for studying vaccine efficacy, as only in this model did nonvaccinated principals develop clinical edema disease. Also, vascular necrosis was much more frequent than in models A and B. However, models A and B were useful for studying vaccine efficacy against a mild, subclinical form of edema disease. Vaccination of suckling piglets with SLT-IIe(E167Q) prevented both clinical edema disease and

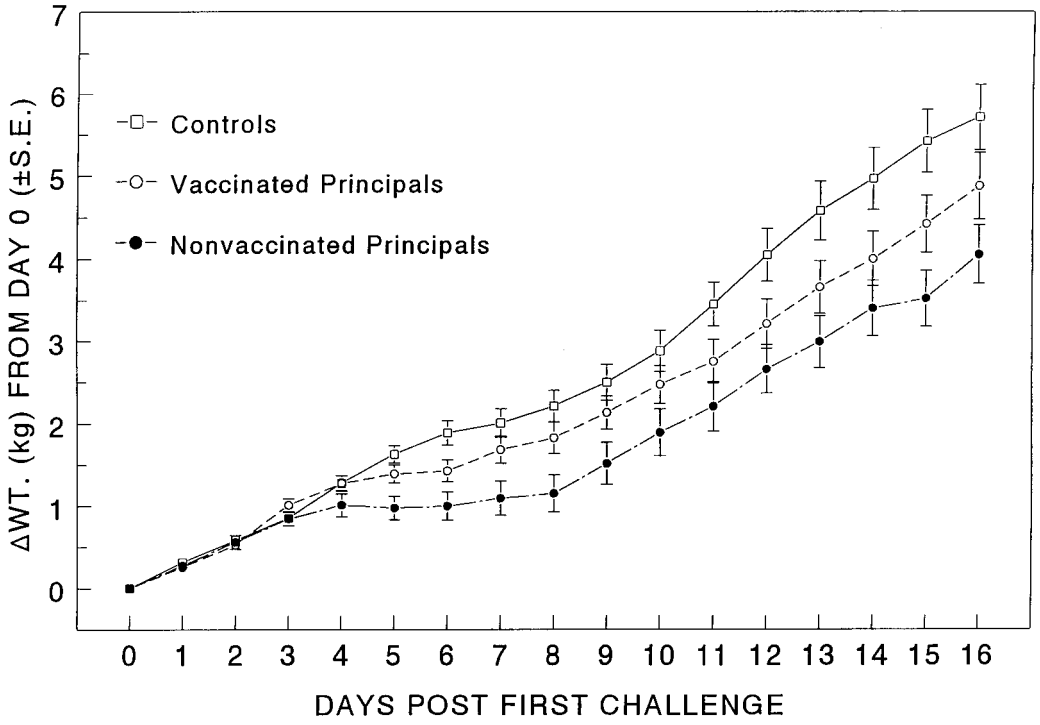

FIG. 2. Effect of vaccination with SLT-IIe(E167Q) and subclinical edema disease on rate of gain in pigs fed a 17% protein diet and inoculated four times. Pigs were either vaccinated with 50 μg of mutant toxin or not vaccinated at 1 and 2 weeks of age. Vaccinated (n = 11) and nonvaccinated (n = 12) principals were orally inoculated at 4, 6, 8, and 10 days postweaning with 10^{10} CFU of strain S1191, and controls (n = 11) were similarly inoculated with strain 123. S.E., standard error.

subclinical edema disease in model C. Moreover, the enzymatic mutant toxin has no negative effects on rate of gain (11) (model A of this study), which is in sharp contrast to the negative effects observed with a toxoid induced by exposure of SLT-IIe to formaldehyde (11).

Serum antibody titers to SLT-IIe induced by vaccination with SLT-IIe(E167Q) varied in the three models in the present study, and these titers were lower than titers obtained in a previous study (11). The vaccination protocols in the three models of the present study and in the previous study were identical. Different genetic backgrounds, nutrition, or other factors may have accounted for these variations in serum antibody titers. An unexpected observation with vaccinated controls in models A and C of the present study was the increase in serum anti-SLT-IIe titers after oral challenge. The reason for this increase after oral challenge with the nontoxigenic strain 123 is unknown and was not investigated. Possibly, exposure of pigs to bacterial lipopolysaccharide caused a generalized stimulation of the immune system which increased serum antibody titers in these two models.

The protein contents of diets in models A and B were similar (17 to 18%), while the diet in model C had a higher protein content (21%). Identical inoculation methods were used in models B and C. The feeds in models B and C were similar with regard to nonprotein factors: energy content (3,200 to 3,300 kcal [ca. 13,400 to 13,800 kJ]/kg), crude fiber content (3%), and fat content (6%). The protein content in feeds in these two models was modified by changing the ratio of soybean meal to ground corn. Host genetic factors play a role in susceptibility to edema disease (5) and could have accounted for the differences in susceptibility in the various models described herein. However, all pigs in models B and C were derived from the same breeding herd and all of these pigs were determined to be genetically susceptible to edema disease on the basis of an in vitro adhesion assay (data not shown) (6). Therefore, diet, not genetics, was likely responsible for the differences in susceptibility to edema disease in models B and C, and this study provides further evidence of the role of diet in susceptibility to edema disease (4, 36).

The data from model C demonstrate that clinical edema disease has a negative effect on both rate of gain and ratio of average daily feed consumption/weight gain. These reductions may be due to direct actions of SLT-IIe, as vaccination with SLT-IIe(E167Q) prevented both the changes in ratio of average daily feed consumption/weight gain and rate of gain. Although no significant differences between treatment groups were observed with respect to rates of gain in models A and B, the rate of gain in nonvaccinated principals was slightly lower than it was for the other experimental groups. The reason for the lack of significant differences in models A and B may be because not all nonvaccinated principals developed vascular necrosis. A decreased rate of gain was observed previously with model A when a majority of the pigs had evidence of vascular necrosis (21). Awad-Masalmeh et al. (1, 2) also observed that pigs which had been inoculated with SLT-IIe-producing *E. coli* had lower rates of gain than pigs experimentally inoculated with SLT-IIe-producing *E. coli* after vaccination. Furthermore, weight gain was reduced in earlier experiments in which pigs were vaccinated with a formalized (partially inactivated) SLT-IIe toxoid (11). Therefore, even mild exposures to SLT-IIe may impact negatively on the rate of gain.

Diarrhea was a common finding with pigs inoculated with strain S1191 in models B and C. The cause of the diarrhea was not determined; however, it was probably caused by strain S1191, as the pigs inoculated with the nonpathogenic *E. coli* strain, 123, did not develop diarrhea. Strain S1191 produces

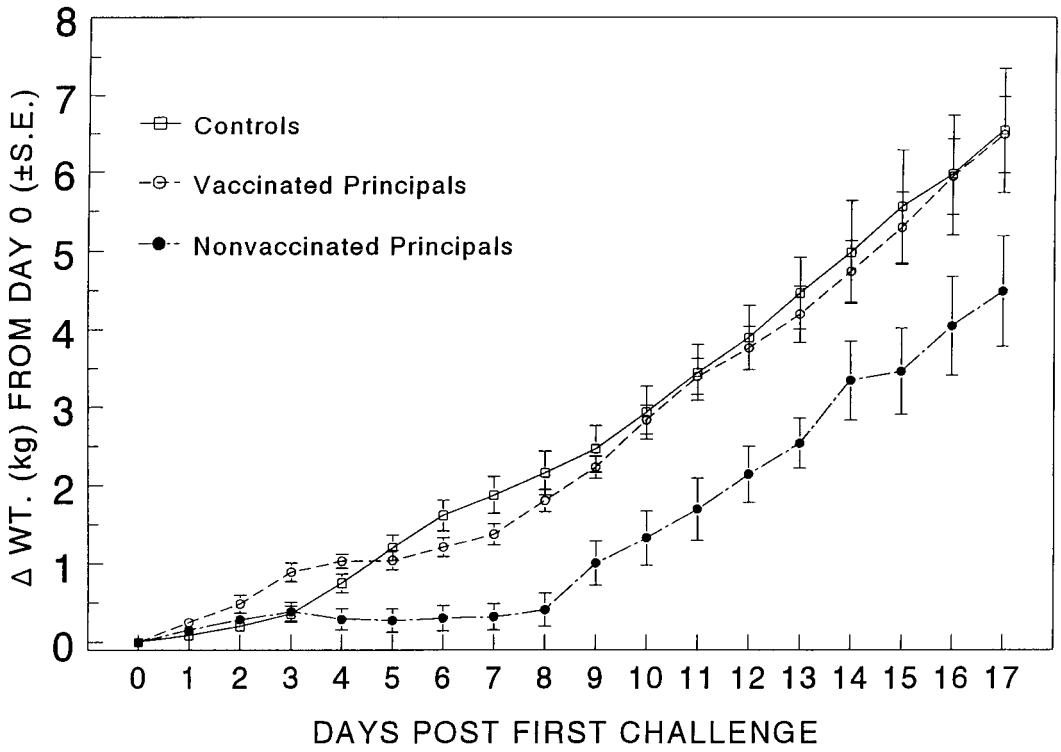

FIG. 3. Effect of vaccination with SLT-IIe(E167Q) and clinical edema disease on rate of gain in pigs fed a 21% protein diet and inoculated four times. Pigs were either vaccinated with 50 μg of mutant toxin or not vaccinated at 1 and 2 weeks of age. Vaccinated (n = 8) and nonvaccinated (n = 11) principals were orally inoculated at 4, 6, 8, and 10 days postweaning with 10^{10} CFU of strain S1191, and controls (n = 11) were similarly inoculated with strain 123. S.E., standard error.

the enterotoxin STb, which would not have been neutralized by antibodies generated by vaccination with SLT-IIe(E167Q).

This study demonstrated that a nontoxic, genetically modified SLT-IIe induced antitoxic immunity that protected pigs against experimental challenge with an SLT-IIe-producing strain of *E. coli*. More importantly, it establishes the potential for using genetically modified, nontoxic Shiga-like toxins for prevention of disease caused by Shiga-like-toxin-producing *E. coli* in other species, such as hemolytic-uremic syndrome in humans.

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